LDA-1: A Ubiquitous Noncollagenous Lamina Densa Component of Basement Membrane Detected by Monoclonal Antibody Technique

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Using monoclonal antibody technology, a new basement membrane antigen, designated as LDA-1, has been identified. This antigen is expressed in all human tissues thus far examined; within skin it is detectable not only within the dermal-epidermal junction but also within dermal vascular and appendageal basement membranes. In addition to human skin, LDA-1 is also detectable within rabbit but not monkey, rat, mouse, guinea pig, or cow skin. This antigen has been ultrastructurally localized to the lamina densa and to a much lesser extent, the adjacent sublamina densa region. ELISA (enzyme-linked immunosorbent assay) revealed no cross-reactivity between LDA-1 and type IV collagen, laminin, fibronectin, and heparan sulfate proteoglycan. In vitro enzymatic studies suggest that LDA-1 is noncollagenous in nature. *J Invest Dermatol 86:286–289, 1986*

Recent studies using both polyclonal and monoclonal antibodies have demonstrated increasing complexity in the antigenic composition of basement membranes [1]. Within the dermal-epidermal junction of human skin, for example, bullous pemphigoid antigen has been localized to the lamina lucida [2–4], and/or adjacent hemidesmosomes [5,6], laminin [7,8], cicatricial pemphigoid antigen [9–11], and fibronectin [12–14] have been identified within the lamina lucida, and type IV collagen [15,16], KF-1 antigen [17–20], epidermolysis bullosa acquisita antigen [21,22], nidogen [23,24], and heparan sulfate proteoglycan [24–26] have each been demonstrated within the lamina densa. In an effort to search for the presence of additional antigens within one or more regions of skin basement membrane, we have utilized hybridoma technology to create a series of monoclonal antibodies having such binding specificity. As a result, we now describe a new noncollagenous lamina densa antigen present in all human tissues thus far examined.

MATERIALS AND METHODS

Source of Tissues Examined The following normal human tissues were obtained either at time of surgery or autopsy by the University of Alabama at Birmingham (UAB) Tissue Procurement Service: buccal mucosa, tongue, upper and lower esophagus, stomach, duodenum, jejunum, ileum, large intestine, rectum, anal canal, kidney, ureter, urinary bladder, urethra, vagina, cervix, uterus, fallopian tube, placenta, trachea, lung, aorta, spleen, lymph node, and skeletal muscle. Normal skin was obtained either from normal adult volunteers or as neonatal foreskin following elective circumcision.

In addition, skin was obtained from the following animals: monkey, mouse, rat, guinea pig, rabbit, and cow.

Preparation of Immunogen Human placenta was chosen for immunogen extraction because of its rich basement membrane content. Fresh tissue was homogenized on ice in the presence of 2 M guanidine in 0.05 M Tris buffer, pH 7.4, and ultracentrifuged (65,000 *g*; 4°C; 1 h). The supernatant was extensively dialyzed against 0.0067 M phosphate-buffered saline, pH 7.4 (PBS), lyophilized, and then stored at −20°C until needed.

Production of LDA-1 Monoclonal Antibody A 12-week-old female BALB/c mouse was immunized s.c. with the above extract (diluted in PBS) in the presence of Freund's complete adjuvant. One week later the animal was given a second dose of the placental extract (in the absence of adjuvant) i.v. and then sacrificed 3 days later. At that time, using standard technique [27,28], the hyperimmune mouse spleen cells were fused with SP 2/0-Ag 14 mouse myeloma cells [29] in the presence of polyethylene glycol 1500 and cultured in Dulbecco's modified Eagle's medium containing fetal calf serum, hypoxanthine, aminopterin, and thymidine. Fourteen days later, hybridoma supernatants were screened by indirect immunofluorescence on human skin for the presence of antibodies directed against basement membrane. Those cell lines showing such reactivity were subsequently expanded, cloned by limiting dilution technique, and reexpanded in the presence of neonatal thymocytes. One such cell line, subsequently designated as LDA-1 on the basis of immunoelectron microscopy findings, was serially recloned to ensure monoclonality and subsequently characterized.

For some of the later studies, monoclonal antibody-rich ascites was prepared in female BALB/c mice following pretreatment i.p. with pristane (Aldrich Chemical, Milwaukee, Wisconsin).

LDA-1 Monoclonal Antibody—Determination of Isotype and *Staphylococcus* Protein A Binding Isotype determination was performed by standard ELISA (enzyme-linked immunosorbent assay) technique [30]. The ability of the LDA-1 monoclonal

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Abbreviations:

EBA: epidermolysis bullosa acquisita

ELISA: enzyme-linked immunosorbent assay

PBS: phosphate-buffered saline, pH 7.4

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antibody to bind *Staphylococcus* protein A was determined by indirect immunofluorescence technique (as described elsewhere) on human skin using sequential incubations of LDA-1 monoclonal antibody and fluorescein-conjugated *Staphylococcus* protein A (Kirkegaard & Perry, Gaithersburg, Maryland).

Characterization of LDA-1 Antigen

ELISA: An ELISA was established for type IV collagen, laminin, fibronectin, and heparan sulfate proteoglycan and was used to examine tissue culture supernatants containing each of the basement membrane-related monoclonal antibodies generated in our laboratories. The assay consisted of serial incubations involving murine monoclonal antibody, horseradish peroxidase-conjugated goat antimouse immunoglobulin, and *o*-phenylenediamine; subsequently optical densities were measured with an ELISA plate reader. Type IV collagen (from human placenta), laminin (from rat yolk sac carcinoma), fibronectin (from human plasma), and heparan sulfate proteoglycan (from rat yolk sac carcinoma) were used as antigenic substrates and were purified as previously reported [26,31–34].

Indirect Immunofluorescence Studies: Human organ and skin species specificities were determined using standard indirect immunofluorescence technique. In brief, 6–8 μm-thick cryostat sections were prepared from freshly embedded specimens from each of these tissues. Following determination of the optimal dilution of monoclonal antibody-rich mouse ascites for the staining of normal human skin and foreskin, serial incubations (room temperature; humidified chamber) were performed on each of the tissues using hyperimmune ascites (diluted in PBS) and fluorescein-conjugated rabbit antimouse IgG (Cappel Laboratories, West Chester, Pennsylvania). Following PBS rinses, each tissue-containing slide was mounted with 50% glycerol in PBS and examined by epi-illumination with a Leitz Laborlux 12 immunofluorescence microscope. Nonhyperimmune mouse ascites, produced by intraperitoneal implantation of nonfused SP 2/0-Ag 14 myeloma cells, was used at the same dilution as a negative control for these studies.

In addition, the same technique was used to assess expression of LDA-1 antigen in the basement membrane matrix-producing rat yolk sac carcinoma.

Immunoelectron Microscopy: Indirect immunoelectron microscopy was performed with the LDA-1 monoclonal antibody using 10 μm-thick cryostat sections of unfixed human foreskin as tissue substrate. Such tissue was serially incubated with LDA-1-rich mouse ascites (diluted 1:10 to 1:40 in PBS containing 1% bovine serum albumin) and γ-chain-specific horseradish peroxidase-conjugated goat antimouse IgG (undiluted or 1:2; Tago, Burlingame, California). Subsequently each slide was washed in 0.2 M Tris HCl buffer, pH 7.6, and incubated in the same buffer containing diaminobenzidine and hydrogen peroxide. Following PBS rinses, each slide was processed for transmission electron microscopy as previously described [35]. As a negative control, nonhyperimmune mouse ascites was substituted for ascites containing LDA-1 monoclonal antibody.

Enzymatic Sensitivity: The relative enzymatic sensitivities of LDA-1 antigen and type IV collagen in intact human foreskin were compared using the following purified enzymatic preparations: bacterial collagenase (form III; 5200 U/ml; Advance Biofactures Corp., Lynbrook, New York), bovine trypsin (1 mg/ml), bovine chymotrypsin (1 mg/ml), and porcine elastase (1 mg/ml), the latter 3 enzymes kindly provided by Dr. Louis Heck (Dept. of Medicine, UAB). For these studies, collagenase was diluted in 0.025 M Tris buffer, pH 7.4, containing 0.01 M $CaCl_2$, while the other 3 enzymes were diluted in 0.05 M Tris buffer, pH 7.6, with 0.15 M NaCl and 0.001 M $CaCl_2$.

Six micron-thick cryostat sections of unfixed human foreskin were prepared as previously noted and preincubated with one of the above-noted enzyme preparations at room temperature (collagenase only) or 37°C for 30 min to 7 h. Subsequently, each specimen was incubated serially with a monoclonal antibody to either type IV collagen (Gay, Fine, unpublished data) or LDA-1 antigen and then with fluorescein-conjugated rabbit antimouse IgG; slides were then mounted and examined by epi-illumination.

RESULTS

Using standard hybridoma technique, an IgG_1 monoclonal antibody-secreting cell line (designated LDA-1) was created; this antibody by indirect immunofluorescence was shown to bind not only to dermal-epidermal but to all basement membranes (vascular and appendageal) within human skin (Fig 1). Using an ascites preparation containing LDA-1 monoclonal antibody, binding was detectable through a titer of 1:10,240. All human organs thus far examined have expressed this antigen within vascular basement membranes and epithelial-connective tissue junctions. In contrast, species specificity was more restricted; LDA-1 antigen was detectable within human and rabbit skin but not within monkey, rat, mouse, guinea pig, or cow skin. LDA-1 antigen was undetectable in rat yolk sac carcinoma, despite the previous demonstration of other basement membrane components (including type IV collagen and laminin) in this tumor [36]. Similar to other murine monoclonal antibodies of the same subclass, LDA-1 did not bind to *Staphylococcus* protein A.

Indirect immunoelectron microscopy demonstrated the presence of LDA-1 antigen within the lamina densa, and to a much lesser extent, within the adjacent sublamina densa region of the basement membrane zone of human skin (Fig 2*A*).

ELISA revealed lack of cross-reactivity of LDA-1 antigen with type IV collagen, laminin, fibronectin, and heparan sulfate proteoglycan.

Whereas type IV collagen was no longer detectable in skin following 3 h of incubation with bacterial collagenase, LDA-1 antigen staining was unchanged after 7 h of exposure. In contrast, expression of each of these 2 lamina densa antigens was similarly diminished or eliminated following 2–4 h of preincubation with elastase, trypsin, and chymotrypsin.

DISCUSSION

To date, 5 distinct components have been identified within the lamina densa of human skin: type IV collagen, KF-1 antigen,

Figure 1. By indirect immunofluorescence, LDA-1 antigen is detectable within the dermal-epidermal junction and within basement membrane surrounding dermal vasculature and appendages (not shown) of normal human skin. *Ep* = epidermis. ×544.

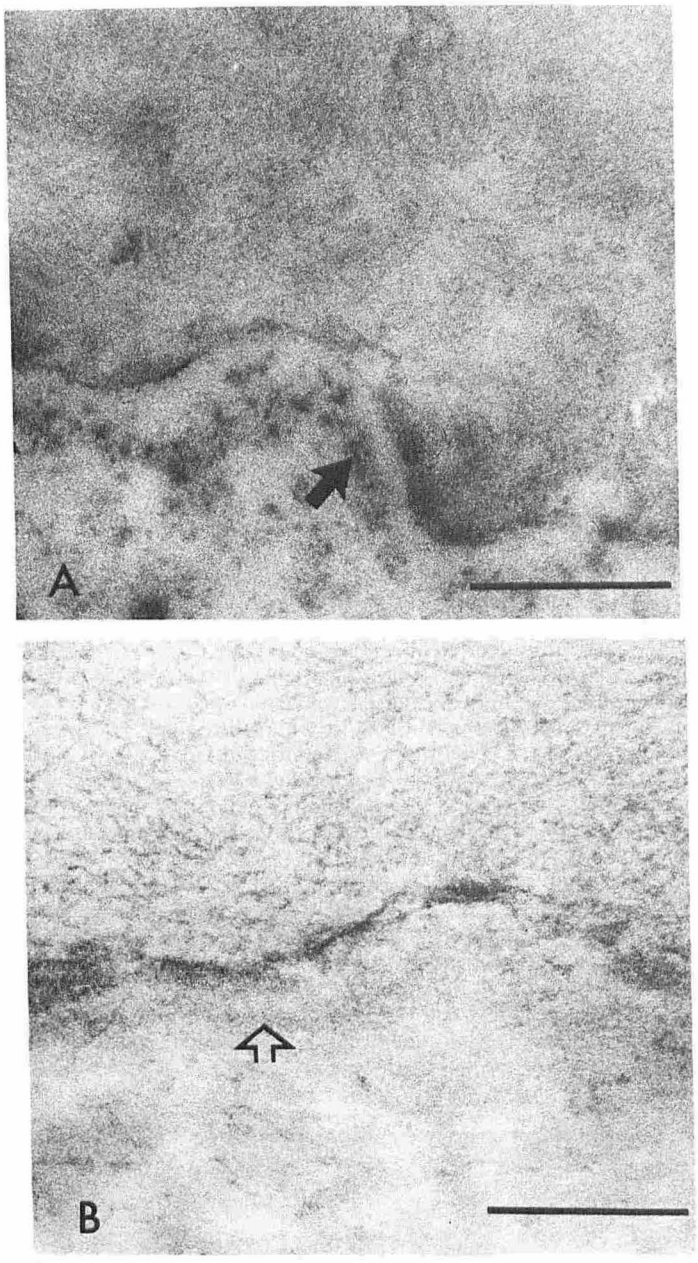

Figure 2. *A*, Immunoelectron micrograph of neonatal foreskin demonstrating localization of LDA-1 antigen (*solid arrow*) to the lamina densa and to a much lesser extent, the sublamina densa region. *Bar* = 0.25 μm. *B*, In contrast to (*A*), no immunoreactants are noted within the lamina densa (*open arrow*) when nonhyperimmune mouse ascites was substituted for ascites containing the LDA-1 monoclonal antibody. *Bar* = 0.25 μm.

epidermolysis bullosa acquisita (EBA) antigen, nidogen, and heparan sulfate proteoglycan. Using monoclonal antibody technology we now report on the detection of a sixth antigen, referred to as LDA-1, within this ultrastructural portion of the basement membrane zone.

LDA-1 monoclonal antibody shows no cross-reactivity by ELISA with 4 of the better characterized basement membrane antigens (type IV collagen, laminin, fibronectin, heparan sulfate proteoglycan) having similar indirect immunofluorescence binding patterns to human skin. Despite the fact that 2 of these antigens, laminin and heparan sulfate proteoglycan, were extracted and purified from nonhuman tissue (i.e., rat yolk sac carcinoma), the lack of any previous evidence of restricted species specificities for these particular antigens suggests that such negative results by ELISA are more consistent with true lack of cross-reactivity rather than a reflection of species specificity. Although ELISAs are as yet unavailable against purified bullous pemphigoid, cicatricial pemphigoid, KF-1, and EBA antigens, the limited expression of the latter 4 antigens in skin (i.e., dermal-epidermal junction) and other tissues (i.e., stratified squamous epithelium, and in some, selected mucosal sites) clearly distinguishes them from LDA-1.

The ultrastructural localization of LDA-1 antigen also distinguishes this antigen from other characterized skin basement membrane antigens. For example, laminin, fibronectin, bullous pemphigoid, and cicatricial pemphigoid antigens are all detectable within skin lamina lucida (or in the case of bullous pemphigoid antigen, possibly within the region of hemidesmosomes) whereas LDA-1 is absent in this region of the basement membrane. In addition, although the remaining skin basement membrane antigens (type IV collagen, EBA antigen, KF-1 antigen, nidogen, and heparan sulfate proteoglycan) have been ultrastructurally localized exclusively to the lamina densa, LDA-1 antigen appears to be present not only within the lamina densa but also to a lesser extent within the upper sublamina densa region, thereby further differentiating it from other known skin basement membrane antigens.

The apparent lack of diminution of expression of LDA-1 antigen in skin following prolonged exposure to bacterial collagenase, in contrast to that observed with type IV collagen, suggests that LDA-1 antigen in noncollagenous in structure. In contrast, its relative lability to elastase, trypsin, and chymotrypsin is similar to that seen with a variety of skin basement membrane components [37].

Another unique feature is the distinctive species specificity noted with the LDA-1 monoclonal antibody when applied to skin. Further studies will be necessary to determine whether these findings reflect the detection of a species-restricted epitope or a new antigen present only in human and rabbit basement membrane.

With regard to the possibility that the LDA-1 monoclonal antibody may be directed against the 7S portion of type IV collagen, this may be discounted for several reasons. First, rat yolk sac carcinoma produces a variety of matrix components including type IV collagen, the latter of which appears to be identical to type IV collagen extracted from human tissues. Second, native human type IV collagen (containing the 7S region) was utilized in our ELISA technique. Therefore, the lack of expression of LDA-1 antigen in this tumor, as determined by indirect immunofluorescence, and the lack of positivity against our type IV collagen preparation by ELISA suggest strongly that the antigenic site recognized by the LDA-1 monoclonal antibody is indeed distinct from the 7S portion of type IV collagen.

It is as yet unknown what role LDA-1 antigen plays in the structural integrity and function of basement membrane. Considering its presence in all human tissue basement membranes, however, it is likely that future studies may demonstrate significant functional interactions between LDA-1 and one or more of the other ubiquitous basement membrane components, such as type IV collagen and laminin.

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